PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION**	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
600.358W001		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/US 99/01236	21/01/1999	22/01/1998
Applicant		
REGENTS OF THE UNIVERSITY OF MINNESOTA et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 3 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [ ] contained in the international application in written form.

   [ ] filed together with the international application in computer readable form.

   [X] furnished subsequently to this Authority in written form.

   [X] furnished subsequently to this Authority in computer readble form.

   [X] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [X] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. _____

   [ ] as suggested by the applicant.

   [ ] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [X] None of the figures.

FOR THE PURPOSES OF INFORMATION ONLY

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/01236

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K7/06 C07K5/10 A61K38/08

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	J.L LAUSER ET AL.: "INHIBITION OF MELANOMA CELL BINDING TO TYPE IV COLLAGEN BY ANALOGS OF CELL ADHESION REGULATOR" J. MED. CHEM., vol. 40, 1997, pages 3077-3084, XP002107028 see abstract ---	1-4, 7-12,14, 23
X	WO 89 01942 A (UNIV MINNESOTA) 9 March 1989 see abstract; table III ---	12,14
X	WO 94 17097 A (UNIV MINNESOTA ;US ARMY (US)) 4 August 1994 see page 1 see page 7, line 25 - line 26 --- -/--	12,14

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 June 1999	12/07/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Cervigni, S

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/01236

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication.where appropriate, of the relevant passages	Relevant to claim No.
X	LASZ E C ET AL: "B3 INTEGRIN DERIVED PEPTIDE 217-230 INHIBITS FIBRINOGEN BINDING AND PLATELET AGGREGATION: SIGNIFICANCE OF RGD SEQUENCES AND FIBRINOGEN AA-CHAIN" BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, vol. 190, no. 1, 15 January 1993, pages 118-124, XP000327808 see abstract see page 123, paragraph 1 ---	12,14
X	EP 0 567 898 A (MANTHEY JUERGEN DR) 3 November 1993 see abstract ---	12,14
X	US 5 382 569 A (CODY WAYNE L ET AL) 17 January 1995 see column 61, line 10 - line 15; claims ---	1-5
X	EP 0 347 890 A (MORISHITA PHARMA ;AJINOMOTO KK (JP)) 27 December 1989 see page 15; example 12; table 15 -----	1,4,6

INTERNATIONAL SEARCH REPORT

information on patent family members

International Application No
PCT/US 99/01236

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 8901942 A	09-03-1989	US 4839464 A	13-06-1989
		US 5019646 A	28-05-1991
		AT 87936 T	15-04-1993
		AU 605637 B	17-01-1991
		AU 2385988 A	31-03-1989
		CA 1305084 A	14-07-1992
		DE 3880139 D	13-05-1993
		DE 3880139 T	21-10-1993
		EP 0366728 A	09-05-1990
		JP 2690767 B	17-12-1997
		JP 3500046 T	10-01-1991
		US 5116368 A	26-05-1992
		US 5171271 A	15-12-1992
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WO 9417097 A	04-08-1994	US 5545620 A	13-08-1996
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		AT 139892 T	15-07-1996
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		US 5433201 A	18-07-1995
US 5382569 A	17-01-1995	AU 679712 B	10-07-1997
		AU 5828094 A	19-07-1994
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		EP 0675902 A	11-10-1995
		JP 8504823 T	28-05-1996
		MX 9308191 A	30-06-1994
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		EP 0584290 A	02-03-1994
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EP 0347890 A	27-12-1989	JP 2004715 A	09-01-1990
		JP 2138952 A	28-05-1990
		JP 2121928 A	09-05-1990
		JP 2799178 B	17-09-1998
		JP 2157230 A	18-06-1990
		DE 68905387 T	21-10-1993
		US 5036052 A	30-07-1990

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Assistant Commissioner for Patents
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in its capacity as elected Office

Date of mailing (day/month/year) 04 October 1999 (04.10.99)	
International application No. PCT/US99/01236	**Applicant's or agent's file reference** 600.358WO01
International filing date (day/month/year) 21 January 1999 (21.01.99)	**Priority date** (day/month/year) 22 January 1998 (22.01.98)
Applicant McCARTHY, James, B. et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

10 August 1999 (10.08.99)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer Patricia Gonzalez
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ATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
110.01130201		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US99/01236	21/01/1999	22/01/1998
International Patent Classification (IPC) or national classification and IPC C07K7/06		
Applicant REGENTS OF THE UNIVERSITY OF MINNESOTA et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 7 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 7 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
10/08/1999	17.05.2000
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Korsner, S-E Telephone No. +49 89 2399 8554

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

Int mational application No. PCT/US99/01236

I. Basis of the report

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

Description, pages:

3-5,9,10,12-15, 17-20	as originally filed				
1,2,6-8,11,16	as received on	12/08/1999	with letter of	10/08/1999	

Claims, No.:

1-23	as originally filed

Drawings, sheets:

1/19-19/19	as originally filed

2. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:
- ☐ the drawings, sheets:

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	13, 15-22, (23)
	No:	Claims	1-6, 7-12, 14
Inventive step (IS)	Yes:	Claims	13, 15-22, (23)
	No:	Claims	1-6, 7-12, 14
Industrial applicability (IA)	Yes:	Claims	1-23
	No:	Claims	

2. Citations and explanations

see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

V. Reasoned statement

The following documents will be referred to in this report:

D1 = US - A - 5 382 569
D2 = EP - A - 576 898 (incorrectly cited as EP-A-567 898 in the ISR; a copy is enclosed)
&
D3 = Derwent Abstract AN 1994-061999 (= JP6016568); enclosed
D4 = Derwent Abstract AN 1995-060335 (= US-A-5380668); enclosed
D5 = J. Jap. Soc. Food Sci. and Techn; 1996, pages 967-969; enclosed

1. Novelty (Article 33(2) PCT)

Among the cited X-documents it appears that only D1 may be relevant.
See the definitions of the hexapeptides as well as the preferred compounds in column 32 (ending in -Ile-Ile-Trp).
It is noted that the first amino acid in the preferred peptides (but not necessarily) is a D-amino acid.
In view of the observation under VIII:2 (below), this must be considered novelty-destroying.

The "corrected" X-document D2 discloses three peptides, (Ia-Ic), which fall under Claims 12 and 14.

In view of the broad scope of Claim 1, i.e. di-, tri-, tetra-, penta- and hexapeptides ending in any combination of (Ile/Val/Leu/...) x (Tyr/Phe/His/Trp/...), see page 5, an additional check has been made by the Examiner in the Derwent Data Base.

The documents D3-D4 (see the marked peptides) were immediately retrieved and no further search was then carried out.
However, a still further document, D5, has been introduced at a late stage of the examination -> see the inhibitors Ile-Tyr, Ile-Trp, Val-Tyr, Val-Trp on page 968.

The purpose of adding D3-D5 is to show that the claimed scope is far too broad.

2. Inventive step (Article 33(3) PCT)

An inventive step objection is raised with regard to the present scope.
The claims are drafted towards a very large number of peptides - but it is not likely that a majority of them can solve the problem [and if not, there is no invention present].
See also the background art referred to on page 6 of the Description.

The Applicant should either restrict the claims to an acceptable scope or else provide further test data to support any larger scope.

Since no further information has been submitted by the Applicant during the international phase, the statement about the inventive step (Box V) is negative except for the specific peptides of Claims 13, 15-22.
Claim 23 will be acceptable once the peptides have been satisfactorily defined.

VIII. Certain observations

Claims:

1.
It should be noted that the drafting of Claims 7-11 does not justify a very broad scope of the independent claim [because it is not the reader who shall carry out the bulk of the work to identify these compounds].

2.
Claim 12 is not clear; it introduces an unclarity as to whether D-amino acids are covered by Claim 1.
Moreover, the term "about" has no clear interpretation and should be deleted in the claim.

3.
The scope of Claim 14 is broader than that of Claim 12.

4.
The scope of Claim 15 extends to 50 amino acids whereas Claim 1 refers to not more than six amino acid residues.
The term "about" should be deleted (see also Claim 22).

5.
Although the drafting of Claim 23 is acceptable, it casts a shadow of a doubt over claims referring to longer peptide sequences.
In other words, it appears that only the short peptides of Claim 1 may be useful in the claimed method?

The peptides should be further defined and the term "about" should be deleted.

Description:

6.
On page 2, line 10, reference is made to peptides up to (about) 100 amino acids. This does not correspond to the claims, which limit the length to (about) 50 in the broadest version.

7.

The statements on page 19, lines 8-11, have no clear interpretation.
It is the claims that define the protection but these should be fairly based on the teaching - and not on any "spirit".

8.

Further prior art documents may have to be identified in the Description; this will depend on later restrictions and has to be settled in the national/regional phase.

PEPTIDES WITH β1 INTEGRIN SUBUNIT DEPENDENT CELL ADHESION MODULATING ACTIVITY

Cross-Referenced to Related Applications

The present application claims priority to U.S. provisional application Serial No. 60/072,119 filed on 22 January 1998, entitled "Peptides with Beta Integrin Subunit Dependent Cell Adhesion Modulating Activity"; U.S. provisional application Serial No. 60/096,212 filed on 12 August 1998 entitled "Peptides with β1 Integrin Subunit Dependent Cell Adhesion Modulating Activity"; and U.S. provisional application Serial No. 60/096,211 filed on 12 August 1998 entitled "Peptides with β1 Integrin Subunit Dependent Cell Adhesion Modulating Activity", the disclosures of which are herein incorporated by reference.

Background of the Invention

Cellular recognition of the extracellular matrix ("ECM") proteins and of other cells has a complex molecular basis, involving multiple distinct cell surface receptors. Integrins are a family of receptors that are fundamentally important for mediating cell adhesion to ECM proteins. Tumor cells adhere to variety of ECM proteins and molecules on other cells as they invade and metastasize. These interactions of tumor cells have a profound effect on their phenotype. although its exact role is complex and not completely understood, α4β1 integrin has been implicated in tumor cell arrest and/or extravasation and is involved in tumor cell invasion and metastasis. This integrin is expressed on many hematopoietic malignancies and also on tumors such as melanomas. α4β1 integrin is unique among integrins in that it binds to both ECM components (e.g. fibronectin) and Ig superfamily adhesion receptors (e.g., VCAM-1) which are expressed on activated endothelial cells and other cell types. α4β1 integrin also binds to itself and promotes homotypic cell adhesion. Although a role for α4β1 integrin has been established in modulating various aspects of tumor cell biology, the mechanisms by which the function of the α4β1 integrin is modulated are complex and not well understood. Understanding the nature of such interactions may help to explain cell-type specific behavior on ECM proteins that are often observed with integrins. There is, accordingly, a continuing need to identify peptides capable of modulating α4β1 dependent cell adhesion as a means of furthering the understanding of the complex interactions involving this integrin.

Summary of the Invention

The present invention relates to peptides capable of modulating β1 integrin subunit dependent cell adhesion. The peptides include a C-terminal amino acid residue having a side chain which includes an aromatic group ("-Ar-") and an amino acid residue with a lipophilic alkyl side chain group ("-Lip-") as the penultimate C-terminal residue. This C-terminal dipeptide sequence is referred to herein as a "LipAr motif." For example, suitable peptides of the invention may include a C-terminal tyrosine residue and an isoleucine residue as the penultimate C-terminal residue, i.e., a C-terminal "IY motif" (Ile-Tyr) . While the present peptides may include a relatively large number of amino acid residues, e.g., up to about 100 amino acid residues or more, as disclosed herein even very small peptides which include the LipAr motif, such as the dipeptide Ile-Tyr and the tripeptide Arg-Ile-Tyr, are capable of modulating β1 dependent adhesion. The present peptides typically have no more than about 50 and, preferably, no more than about 25 amino acid residues. The LipAr C-terminated peptides are preferably capable of inhibiting the β1 integrin subunit dependent adhesion of cells, such as the α4β1 integrin dependent adhesion of Ramos cells and the α5β1 integrin dependent adhesion of erythroleukemic cells (e.g., the erythroleukemic cell line K562).

Brief Description of the Drawings

Figure 1 shows a graph of % adhesion of 8A2 stimulated Ramos cells to IIICS-GST as a function of the concentration of a number of alanine knockout analogs of FN-C/H V+Y. FN C/H V+Y and a scrambled variant lacking a C-terminal IY motif ("sV"; RPQIPWARY (SEQ ID NO:2)) were included as controls.

Figure 2 shows a graph of % adhesion of 8A2 stimulated Ramos cells to IIICS-GST as a function of the concentration of a number of alanine knockout analogs of FN-C/H V+Y. FN C/H V+Y and its scrambled analog sV were included as controls.

Figure 3 shows a graph of % adhesion of 8A2 stimulated Ramos cells to IIICS-GST as a function of the concentration of a number of fibronectin fragments tagged with a C-terminal tyrosine residue. FN C/H V+Y and its scrambled analog sV were included as controls.

reported to inhibit the binding of peripheral blood mononuclear cells and spleen cells to fibronectin and endothelial cell monolayers (see, e.g., Wahl et al., J. Clin. Invest., 94, 655-662 (1994)). Two of these peptides, FN-C/H I+Y and FN-C/H V+Y, contain a C-terminal LipAr motif. The amino acid sequence of FN-C/H I+Y is YEKPGSPPREV-VPRPRPGVY (SEQ ID NO:38). The amino acid sequence of FN-C/H V+Y is WQPPRARIY (SEQ ID NO:l). The other two Tyr-tagged fibronectin C-terminal heparin binding domain related peptides do not contain a C-terminal LipAr motif (both peptides end in "TY" (Thr-Tyr)). The amino acid sequences of the these other two fibronectin C-terminal heparin binding domain fragments are KNNQKSEPLIGR-KKTY (FN-C/H II+Y; (SEQ ID NO:39)), and SPPRRARVTY (FN-C/H IV+Y; (SEQ ID NO:40)). Although all four Y-tagged fragments inhibit leukocyte adhesion to fibronectin *in vitro*, only three of the four, FN-C/H I+Y, FN-C/H II+Y and FN-C/H V+Y, are reported to exhibit anti-inflammatory properties in an *in vivo* rat model. One of the four, FN-C/H V+Y, has also been reported to have to inhibit adhesion to VCAM, another extracellular matrix protein. The reported results suggest that the biological activity of the Y-tagged fibronectin C-terminal heparin binding domain fragments is a functional of the specific sequence of each of the peptides.

Several analogs were prepared to examine whether the inhibition of the β1 integrin dependent cell adhesion is effected by the chirality of the inhibitor. The all D-form of FN-C/H V+Y (SEQ ID NO:1) and the all L-form of retro inverso FN-C/H V+Y (SEQ ID NO:40; the all L-form of YIRARPPQW, the reverse primary sequence of FN-C/H V+Y) were prepared and examined in the 8A2 stimulated Ramos cell adhesion assay. Neither of these two compounds inhibited Ramos cell binding, suggesting that the present peptides preferably include the C-terminal LipAr motif in the form of L-enantioneric amino acid residues.

It has surprisingly been discovered, however, that the alanine knockout analogs of FN-C/H V+Y which preserve the C-terminal LipAr motif (i.e., retain the C-terminal Ile-Tyr dipeptide sequence) are capable of inhibiting β1 integrin dependent cell adhesion. As used herein, the term "alanine knockout analog" refers to an analog of a peptide in which a single residue has been substituted by an alanine residue. Two of the alanine knockout analogs of FN-C/H V+Y have an alanine

residue substituted for one of the arginine residues in the "PRARI" motif (Pro-Arg-Ala-Arg-Ile (SEQ ID NO:41)) within FN-C/H V+Y which has previously demonstrated to be the implicated in stimulated focal contact formation (see, e.g., Woods et al., Molec. Biol. Cell, 4, 605-613 (1993)). These alanine knockout analogs have the amino acid sequences WQPPRAAIY (SEQ ID NO: 8) and WQPPAARIY (SEQ ID NO: 17). Two of the other alanine knockout analogs, AQPPRARIY (SEQ ID NO: 3), WAPPRARIY (SEQ ID NO: 4), also differ from FN-C/H V+Y by a non-conservative amino acid substitution (Ala for Trp and Ala for Gln respectively).

As the examples described herein demonstrate, peptides which differ from FN-C/H V+Y by a non-conservative amino acid substitution but retain the C-terminal LipAr motif can be capable of modulating β1 integrin subunit dependent cell adhesion even if the overall physical properties of the peptide differ substantially from FN-C/H V+Y. For example, an FN-C/H V+Y analog in which the two arginine residues have been replaced by aspartic acid residues inhibits the 8A2 stimulated adhesion of Ramos cells at least as strongly as FN-C/H V+Y. The analog, WQPPDADIY (SEQ ID NO: 38), exhibits this activity even though it has an overall net charge of -2 (in contrast to the +2 net charge of FN-C/H V+Y).

Even more surprising than the fact that non-conservative substitution variants of FN-C/H V+Y retain the capability of inhibiting β1 integrin subunit dependent cell adhesion, is the fact that other short Lip Ar C-terminated peptides with little or no sequence homology to FN-C/H V+Y also possess this type of biological activity. The results disclosed herein establish that even peptides with less than 50% homology with the corresponding C-terminal portion of FN-C/H V+Y or FN-C/H I+Y exhibit the capability of inhibiting β1 integrin subunit dependent adhesion. Examples of such peptides include ARITGYIIY (SEQ ID NO:14), RARITGYIY (SEQ ID NO:13), PRQAWRPIY (SEQ ID NO:18), and RPAPQRWIY (SEQ ID NO:20).

As used herein, the term "% homology" refers to the percentage of amino acid residues of a peptide which are either identical to that of an original peptide sequence or differ from the original peptide sequence solely as a result of a conservative amino acid substitution. For example, the peptide PAIFDRSCGS has

40% identity and 80% homology with respect to the peptide sequence PKVMERTCDS.

For the purposes of this invention, conservative amino acid substitutions are defined to result from exchange of amino acids residues from within one of the following classes of residues: Class I: Ala, Gly, Ser, Thr, and Pro (representing small aliphatic side chains and hydroxyl group side chains); Class II: Cys, Ser, Thr and Tyr (representing side chains including an -OH or -SH group); Class III: Glu, Asp, Asn and Gln (carboxyl group containing side chains): Class IV: His, Arg and Lys (representing basic side chains); Class V: Ile, Val, Leu, Phe and Met (representing hydrophobic side chains); and Class VI: Phe, Trp, Tyr and His (representing aromatic side chains). The classes also include related amino acids such as 3Hyp and 4Hyp in Class I; homocysteine in Class II; 2-aminoadipic acid, 2-aminopimelic acid, γ-carboxyglutamic acid, β-carboxyaspartic acid, and the corresponding amino acid amides in Class III; ornithine, homoarginine, N-methyl lysine, dimethyl lysine, trimethyl lysine, 2,3-diaminopropionic acid, 2,4-diaminobutyric acid, homoarginine, sarcosine and hydroxylysine in Class IV; substituted phenylalanines, norleucine, norvaline, 2-aminooctanoic acid, 2-aminoheptanoic acid, statine and β-valine in Class V; and naphthylalanines, substituted phenylalanines, tetrahydroisoquinoline-3-carboxylic acid, and halogenated tyrosines in Class VI.

In another embodiment of the present invention, the peptides contain no more than 10 amino acid residues and have a sequence which does not correspond substantially to the amino acid sequence of FN-C/H V+Y. As used herein, the sequence of a particlar peptide does not correspond substantially to a reference amino acid sequence, if the particular peptide sequence has less than about 80% identity and preferably less than about 50% homology with the reference sequence.

One group of particularly suitable peptides of the invention are those which include a C-terminal "IIY" motif, i.e., the sequence of the three C-terminal most amino acid residues is Ile-Ile-Tyr. One such peptide contains 9 amino acid residues and has the sequence ARITGYIIY (SEQ ID NO:14).

From a variety of standpoints, including cost, ease of production and overall efficiency, smaller versions of the present peptides can offer many distinct

The invention will be further described by reference to the following detailed examples. The examples are meant to provide illustration and should not be construed as limiting the scope of the present invention.

Examples

Assay for Inhibition of α4β1 Dependent Cell Adhesion

The assay described below was performed to determine whether specific peptides were capable of inhibiting β1 integrin subunit modulated cell adhesion and, in particular, of inhibiting α4β1 dependent Ramos cell adhesion to IIICS-GST, an α4β1 ligand. IIICS-GST is recombinantly produced fusion protein which contains a fragment from the type III CS region ("IIICS") of plamsa fibronectin fused to glutathione-S-transferase ("GST"). The fibronectin fragment corresponds to fibronectin amino acid residues 1961 to 2039 (sequence numbering for fibronectin as designated in U.S. Patent 4,839,464) and includes the DELPQLVTLPHPNLHGPEILDVPST (SEQ ID NO:29) amino acid sequence ("CS1"; fibronectin residues 1961-1985). A synthetically prepared peptide having the CS1 sequence has been shown to interact with α4β1 integrin on human lymphocytes and promote cell adhesion but does not bind to heparin. In the assay, a 96-well plate was coated with the substrate IIICS-GST. Ramos cells stimulated with the β1 activating monoclonal antibody 8A2 ("Ab 8A2") were preincubated with one of the peptides to be evaluated for their ability to adhere to IIICS-GST.

The fusion protein can be constructed by first using PCR primers to amplify the coding sequence for residues 1961-2039 of plama fibronectin. The PCR product can be introduced into a suitable bacterial expression vector in frame with the gene for GST. The resulting vector can be transformed and expressed in a suitable host cell, such as *E.Coli*, to produce the fusion protein. If desired, the fusion protein can be purified using a glutathione column. In control experiments in which GST alone was coated onto a 96-well plate, no adhesion of 8A2 activated Ramos cells was observed.

A 96-well plate was coated in triplicate with 50 μl/well of IIICS-GST diluted to 3-5 μg/ml in PBS containing lmM $CaCl_2$, $MgCl_2$ ("PBS/cations") and incubated overnight at 37°C. The IIICS-GST solution was removed and the wells were

are shown in Figure 13. Peptides with the "IY" motif at the N-terminus, IYWQPPRAR (SEQ ID NO:34), or in the middle of the peptide, WQPIYPRAR (SEQ ID NO:35) were inactive in the assay. Switching the order of the Ile and Tyr residues at the C-terminus of an FN C/H V+Y analog, WQPPRARYI (SEQ ID NO:35), also resulted in a peptide which was inactive in the $\alpha 4\beta 1$ dependent Ramos cell adhesion inhibition assay. Finally, control peptide having the tyrosine tag removed from the C-terminus of FN C/H V+Y, WQPPRARI (SEQ ID NO:35), was also inactive in the assay.

Example 9 - Inhibition of Adhesion by a Negatively Charged "LipAr" Peptide

All the the LipAr terminated peptides described in the above examples which were active in the Ramos cell adhesion inhibition assay have a net positive charge. In order to determine whether a net positive charge is required for this activity, an FN-C/H V+Y analog in which the 2 arginines (positively charged) were replaced by aspartic acid residues (negatively charged) was evaluated. Importantly, the C-terminal "LipAr" motif ("IY") was retained in this peptide, WQPPDADIY (SEQ ID NO: 38). Figure 14 clearly demonstrates that substitution of the arginines with aspartic acid residues does not alter the ability of the peptide to inhibit $\beta 1$ integrin subunit dependent adhesion, thereby further demonstrating the importance of the "LipAr" motif to this activity.

Example 10 - Inhibition of adhesion by PRARIY versus PRARI

In an experiment which further demonstrated the correlation of a C-terminal LipAr motif with $\beta 1$ integrin subunit dependent adhesion adhesion, the peptide PRARIY (SEQ ID NO: 24) and the corresponding sequence lacking the terminal aromatic residue ("Tyr") were evaluated for their ability to inhibit adhesion in the Ramos cell assay. Consistent with the previous results demonstrating the requirement for a C-terminal "LipAr" motif, PRARIY but not PRARI was able to inhibit $\alpha 4\beta 1$ mediated Ramos cell adhesion to IIICS-GST (see Figure 15).